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ADP-ribosylation factors regulate the development of CT signaling in immature human enterocytes

Lei Lu,[1] Abdullah Khan,[2] and W. Allan Walker[1]

[1]*Developmental Gastroenterology Laboratory, Massachusetts General Hospital for Children, Boston, Massachusetts;*
[2]*The Aga Khan University Hospital, Karachi, Pakistan*

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Lu L, Khan A, Walker WA. ADP-ribosylation factors regulate the development of CT signaling in immature human enterocytes. *Am J Physiol Gastrointest Liver Physiol* 296: G1221–G1229, 2009. First published April 9, 2009; doi:10.1152/ajpgi.90686.2008.—Diarrheal disease is a major cause of morbidity and mortality in infants and children worldwide. Evidence suggests that the interaction of immature human enterocytes with bacteria and their enterotoxins may account for the increased susceptibility of neonates to diarrheal diseases. However, the precise mechanisms that contribute to the excessive response to cholera toxin by the immature gut are largely unknown. Our aim was to characterize the cellular/molecular changes in Gs_α during gut development. In this study, a colonic human epithelial cell line (T84) was used as representative of a mature enterocyte and a human fetal primary small intestinal cell line (H4) as representative of an immature enterocyte. Using our cell culture model of human intestinal development, we provide consistent evidence that cholera toxin (CT)-mediated Gs_α activation in fetal enterocytes differs from that of mature enterocytes, and the difference may be related to ADP-ribosylation factor (ARF) interaction with the CT-signaling process. Here we demonstrated that ARF1 may play a critical role in clathrin-mediated CT trafficking through the endoplasmic reticulum and Golgi and that ARF6 may facilitate clathrin-mediated CT endocytosis that leads to enhanced Gs_α activation by CT. Collectively, these findings support our hypothesis that there is a developmentally regulated intestinal cellular response to bacterial exotoxins involving complex cellular events that accounts for the increased incidence and severity of toxogenic diarrhea during infancy.

cholera toxin trafficking; developmental cholera toxin-Res; stimulatory guanine nucleotide binding protein-α

CHOLERA TOXIN (CT) is a pentavalent protein comprised of five binding (B) subunits that interact with an enterocyte microvillus glycolipid ganglioside (GM1) and one active (A) subunit with enzymatic activity that is capable of ADP-ribosylation of the α-subunit of a heterotrimeric guanine nucleotide-binding (G) protein Gs (Gs_α) (16). In polarized epithelial cells, the activation of signal transduction by CT requires endocytosis of the toxin-receptor complex into the apical endosome, its translocation by retrograde transport into the Golgi cisternae or endoplasmic reticulum (ER), and finally the movement of the toxin to its site of action in the basolateral membrane (reviewed in Refs. 18, 21, 37).

CT exerts its effect on intestinal cells via ADP-ribosylation of the α-subunit of a heterotrimeric guanine nucleotide binding (G) protein Gs, the stimulatory G protein for adenylate cyclase (AC). After ribosylation, Gs_α remains activated and causes a 100-fold increase in cAMP production (37), which can lead to excessive chloride secretion and a severe secretory diarrhea in humans (9).

The discovery of CT and its mechanism of interaction with the enterocyte as a prototypic bacterial exotoxin has yielded vital information about the pathophysiology of toxogenic diarrhea and CT intracellular signal transduction steps, including the role of G proteins in excessive chloride secretion (18, 19, 38). These studies have led to the discovery and characterization of a family of small GTP-binding proteins called ADP-ribosylation factors (ARFs) named for their ability to enhance the CT-catalyzed ADP-ribosylation of Gs_α as an allosteric activator of the toxin (29, 30). In addition, ARFs have been recently recognized to play a critical role in vesicular membrane trafficking (30).

There is increasing evidence that an immature enterocyte interaction with bacteria may, in part, explain the increased susceptibility of neonates to infectious diarrhea (4, 14, 40). This laboratory has studied the excessive secretory response to CT in animal models and has concluded that the principal mechanism is mediated by a developmentally regulated inappropriate postreceptor response (5, 20, 41, 43). For example, a previous study has shown that Gs_α expression is upregulated in preweaned compared with postweaned and adult rat enterocytes (41). This developmental change in Gs_α might be a key factor in mediating the excessive secretory response to CT in the young rat (41).

Recently we have extended these observations to experimental models of human intestinal development (23, 25). In these studies, we have also provided evidence that CT induces an enhanced secretory reaction mediated in part by a developmental upregulation of the cAMP response in immature vs. mature human enterocytes (23), and this enhanced response to CT is largely due to an excessive uptake of CT by a developmentally regulated clathrin-endocytic pathway (25). In this study, we examined the cellular/molecular basis for CT signaling in the developing human gut and provide evidence that ARFs are involved.

MATERIALS AND METHODS

Materials. CT was purchased from Calbiochem (EMD, La Jolla, CA). Anti-clathrin heavy-chain (Cla-HC) antibody (mAb) was purchased from BD Transduction Laboratories (Palo Alto, CA). Anti-Gs_α and anti-$G_{\beta\gamma}$-complex rabbit pAb (anti-$G_{\beta\gamma}$) were obtained from Calbiochem. Anti-ARF1 and 6 mAb were obtained from Abcam (Cambridge, MA). Anti-CT-A and B polyclonal antibodies were obtained from Dr. Wayne Lencer's Laboratory (Children's Hospital, Boston, MA). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies are from Bio-Rad (Hercules, CA). Hemaglutinin (HA)-tagged full-length ARF1 and ARF6 plasmids were kindly provided by Dr. Jim Casanova (Department of Cell Biology, University xof Virginia, Charlottesville, VA). [^{32}P]NAD was obtained from

Address for reprint requests and other correspondence: L. Lu, Developmental Gastroenterology Lab., Massachusetts General Hospital for Children, 114 16th St. (114-3503), Charlestown, MA 02129-4404 (e-mail: lul@helix.mgh.harvard.edu).

PerkinElmer (Waltham, MA). ARF inhibitory peptide (ARFI) was obtained from Biomol (Plymouth Meeting, PA). Human recombinant EGF, Trizol, SuperScript III Platinum SYBR Green One-Step qRT-PCR kits, Stealth siRNA duplex oligoribonucleotides for ARF1, 3, 4 and 6, and lipofectamine RNAi MAX were obtained from Invitrogen (Carlsbad, CA). All tissue culture media and reagents are obtained from Invitrogen-GIBCO. All other chemicals unless specified were purchased from Sigma-Aldrich (St. Louis, MO).

Cell cultures. H4 (fetal) and T84 cells (adult) were used in this study. H4 cells have been used as an immature human intestine model in previous studies (6, 25, 31, 39). H4 cells were routinely maintained in DMEM supplemented with 10% FBS and human recombinant insulin (0.5 U/ml). In selected experiments, H4 cells were preincubated in H4 media containing 1 μM hydrocortisone (HC) for 5–7 days (H4/HC) before further treatment. T84, a well-characterized and highly differentiated human colon carcinoma cell line, has been used as a mature human intestinal model (6, 25, 31, 39). T84 cells (American Type Culture Collection, Rockville, MD) were maintained in DMEM-F12 Ham with 5% FBS. Cells were seeded on either 12-mm (24-well) or 33-mm (6-well) tissue culture plates or permeable inserts. T84 cells, grown on permeable inserts, were used when they became confluent and formed polarized monolayers and tight junctions (e.g., resistance $\Omega \geq 1000$). In some experiments, T84 cells, grown on permeable inserts, were used before cells reached confluence (subconfluent, $\Omega < 100$). These cells have neither formed tight junctions nor polarized monolayers. CT was used at a 20 nM concentration (dose range 0.2–20 nM). All CT-stimulation experiments were done in serum-free HBSS supplemented with 0.25% bovine albumin.

Determination of the developmental Gs_α and ARF gene expression. The level of Gs_α and ARF mRNA was measured in duplicate for each sample by quantitative RT-PCR (qRT-PCR) using a SYBR Green One-Step qRT-PCR kit. The level of Gs_α and ARF mRNA expression was normalized to the standard GAPDH level for each sample.

Determination of CT-catalyzed ADP-ribosylation of Gs_α. In vitro [^{32}P]ADP-ribosylation was carried out in H4 and T84 cell lysates using the method described by McKenzie (27). Briefly, H4 and T84 cells were harvested in lysis buffer containing 0.5% SDS. A sample (20 μg) of cell lysates was assayed in a 50-μl volume containing [^{32}P]NAD (3 μM, 4×10^6 counts/min), potassium phosphate buffer (250 mM, pH 7.0), thymidine (20 mM), ATP (1 mM, pH 7.0), arginine hydrochloride (20 mM), and 100 μM GTP. CT was activated by incubation with 50 mM DTT for 60 min at room temperature and then added to the mixture at a final concentration of 50 μg/ml. The assay was carried out in a water bath at 37°C for 60 min and terminated by transfer to ice. Next, after in vitro ADP-ribosylation, the samples were prepared for SDS-PAGE by sodium deoxycholate/trichloroacetic acid precipitation as detailed elsewhere (27) and visualized by autoradiograph. In selected experiment, 100 μg of fresh rat brain extract was added to the sample before the assay was carried out as described above.

Immunoprecipitation, SDS-PAGE, and Western blot (immunoblot analysis). After experiments were completed, cells were rinsed with HBSS. Surface-bound toxin was removed with acidified HBSS (pH 2.5). Cells were lysed in Tris-buffered solution (4°C) containing 1% Triton X-100 (1% TTBS), 0.5% SDS, and protease inhibitors as described previously (25). In some experiments, Triton-soluble proteins were collected first, and the insoluble fraction was then collected in 1% TTBS containing 0.5% SDS. Both fractions (10 μg proteins) were analyzed by SDS-PAGE (4–20% linear gradient gel) and Western blotting. For immunoprecipitation, H4 and H4/HC cells were transfected with HA-tagged ARF1 and ARF6 expression vectors as well as the vector alone as a control. Seventy-two hours after transfection, cells were washed with HBSS(+) and then harvested in lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1.0% Nonidet P-40, 10% glycerol, 0.5 mM PMSF, and a complete protease inhibitor tablet (Roche) and then extracted for 15 min on ice. Cell lysates were then incubated for 2 h (4°C) with monoclonal anti-HA antibody (1 μg/IP) to allow for binding of antibody to antigen. After incubation, 30 μl (~50% slurry) of protein A Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) to the lysate and incubated on a rotating device at 4°C for 60 min. The protein A beads were collected by spinning briefly and washed with lysis buffer four times before mixing with SDS sample buffer containing reducing agent. The mixture was then boiled for 3–5 min before SDS-PAGE and Western blot analysis.

Briefly, lysates were resolved on a 4–20% linear gradient polyacrylamide gel and transferred onto nitrocellulose membranes. After being blocked with 5% nonfat milk solution, membranes were sequentially probed with appropriate dilution of primary (1:1,000) and secondary antibodies (1:5,000) and visualized by enhanced chemiluminescence (Bio-Rad). GAPDH was used as a housekeeping protein to control for equivalent loading.

Sucrose equilibrium density centrifugation. Confluent monolayers of T84, H4, and H4/HC cells were used for isolation of detergent-insoluble glycolipid/rafts. All steps were completed at 4°C as described in detail elsewhere (2, 50). Briefly, cells were lysed in 1% Triton X-100 (1% TBS) and homogenized with 15 strokes in a Dounce homogenizer on ice. The subsequent homogenates were adjusted to a 40% sucrose concentration with an equal volume of 80% sucrose in TBS, layered under a linear 5–30% or discontinuous sucrose gradient, and centrifuged at 39,000 revolution/min for 16–20 h in a swinging bucket rotor (model SW 41). Ten fractions were collected sequentially from the top, and 20 μl of each fraction was analyzed by SDS-PAGE and Western blotting.

RNA interference. H4 and H4/HC cells were plated on 6- or 24-well plates at an optimal density and then transfected with small interference RNA (siRNA) using lipofectamine (Invitrogen). ARF1, ARF3, ARF4, and ARF6 (stealth select, Invitrogen) and a scrambled control siRNA (at a dose range 0.1–100 nM for initial study and optimal concentration afterward) were used in selected experiments. After transfection, cells were incubated for 48–72 h before further study.

Expression of HA-ARF1 and 6 DNA. The expression vector (1 μg DNA) containing full-length HA-tagged ARF1 and ARF6 as well as vector alone were transfected into H4, H4/HC, and T84 cells using lipofectamine agent LTX according to manufacturer's recommendation. Seventy-two hours after transfection, cells were exposed to CT or EGF before cell lysates were harvested for cAMP and Western blot analysis.

Enzyme immunoassay. We used cAMP levels as the functional indicator for the CT response in this study. Enzyme immunoassays (EIAs) were used to measure cAMP levels as described previously (23).

Statistical analysis. Data were analyzed by two-sided Student's *t*-test using Microsoft Excel and expressed as means ± SE ($n = 4–6$).

RESULTS

Differential expression and activation of Gs_α. Previous studies from our laboratory demonstrated that there is an increase in Gs_α mRNA expression and an excessive Gs_α ribosylation in preweaned compared with postweaned rat enterocytes (41). However, little is known about the gene expression and regulation of Gs_α in human enterocytes. Before investigating the cellular mechanism for CT activation of Gs_α, we examined its expression in H4 vs. T84 cells. No significant difference was noted in Gs_α mRNA expression between H4 and T84 cells. Real-time, semi-quantitative PCR indicated that Gs_α mRNA is highly expressed in both cells, but the expression level is slightly higher in T84 compared with H4 (Fig. 1*A*). Next, we examined the cellular distribution of Gs_α. In H4 cells, Gs_α is present principally in the detergent-soluble membrane (non-raft) fraction, whereas in T84 cells Gs_α is largely present in the detergent-insoluble and lipid raft-associated membrane frac-

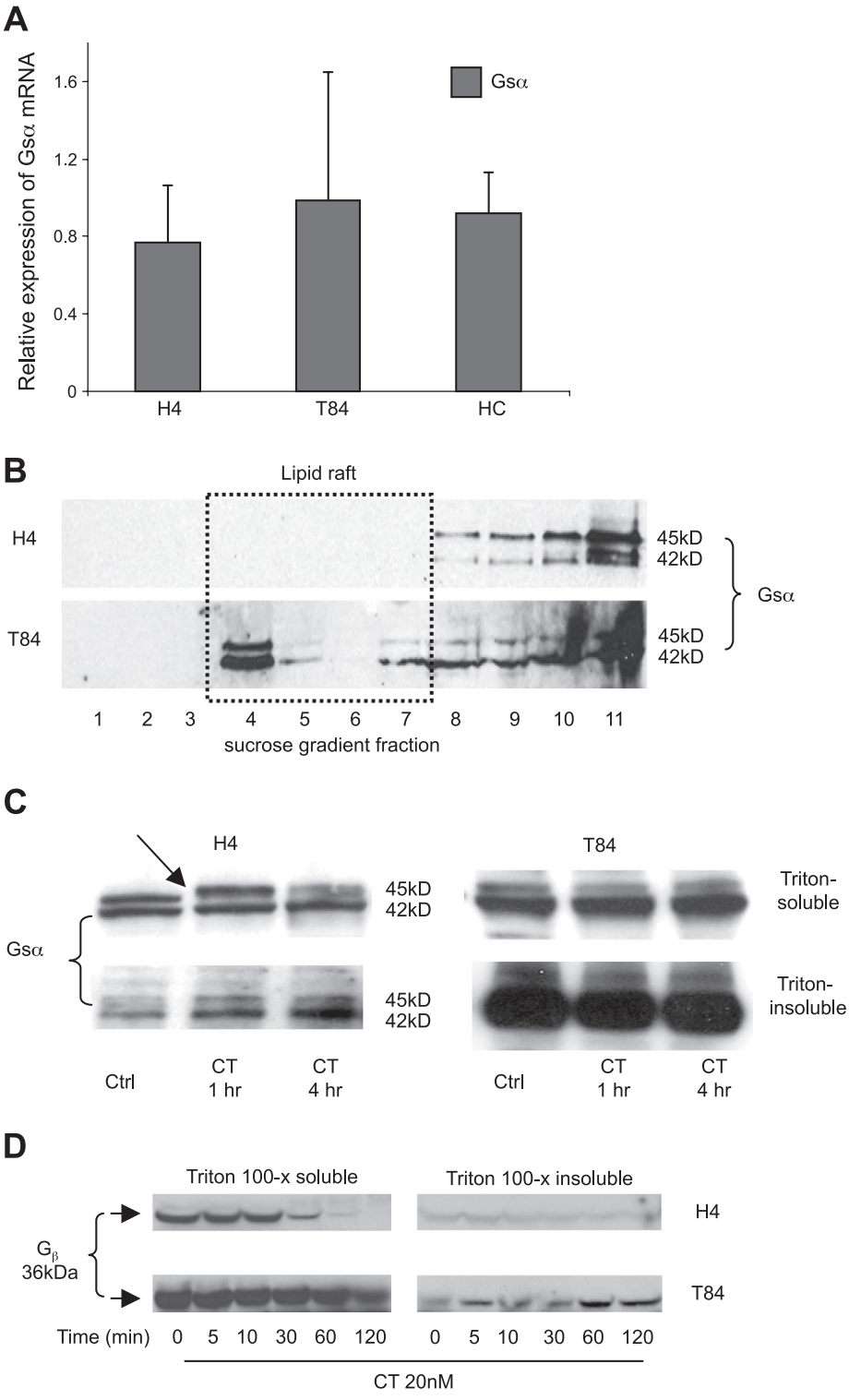

Fig. 1. Heterotrimeric G protein distribution and activation in H4 vs. T84 cells. *A*: real-time PCR of Gs_α mRNA expression in H4, T84, and H4/hydrocortisone (HC) cells normalized by GAPDH expression. *B*: Western blots (4–20% gel) depicting Gs_α protein distribution on a sucrose gradient (5–30%) column probed with an anti-Gs_α (COOH terminus) antibody (1:1,000 dilution). *Fractions 4–7* (box) are lipid-raft fractions. *C*: in H4 cells Gs_α is present principally in Triton X-100-soluble membrane fraction, whereas in T84 cells Gs_α is largely present in Triton X-100-insoluble fraction. Cholera toxin (CT)-induced activation of Gs_α differs in H4 vs. T84 cells (the arrow indicates a retarded migration of Gs_α isoform) (45 kDa) on SDS-PAGE (4–20% linear gradient gel) attributable to ADP-ribosylation. Ctrl, control. *D*: Western blot analysis of $G_{\beta\gamma}$ in H4 vs. T84 cells probed with an anti-$G_{\beta\gamma}$ complex pAB (1:1,000). $G_{\beta\gamma}$ is present principally in Triton X-100-soluble membrane fraction in both H4 and T84 cells. After CT exposure, $G_{\beta\gamma}$ is dissociated from the membrane fractions in H4 cells in a time-dependent manner, but this is absent in T84 cells.

tions (Fig. 1*B*). In addition, Gs_α protein levels were higher in T84 compared with H4 cells in both cellular fractions (Fig. 1*C*). However, after CT exposure, Western blot analysis demonstrated a higher CT-induced Gs_α activation in H4 compared with T84 cells, as indicated by a slower migration of the 45-kDa Gs_α isoform on a 4–20% linear gradient SDS-PAGE gel attributable to the ADP-ribosylation of Gs_α (Fig. 1*C*, arrow). It is hypothesized that Gs_α activation is necessarily accompanied by subunit dissociation in situ. We next compared the $G_{\beta\gamma}$ cellular distribution after CT intoxication in H4 vs. T84 cells. In H4 and T84 cells, $G_{\beta\gamma}$ was principally localized in the detergent-soluble membrane fraction. Furthermore, upon CT exposure, there was a decrease of membrane-associated $G_{\beta\gamma}$ in H4 cells but not in T84 cells (Fig. 1*D*).

Previously, we have shown a CT-induced excessive cAMP response in immature human enterocytes, indicating an exces-

sive activation of AC (23). We have also shown that immature enterocytes have a significantly higher rate of CT endocytosis compared with mature enterocytes, and this may contribute to the excessive CT-catalyzed Gs_α activation in these cells (25). We next investigated in T84 cells the CT dose responsiveness under subconfluent (nonpolarized) and confluent (polarized) conditions and compared this response to that in H4 cells with or without HC. Polarized T84 cells can form tight junctions and thus separate the apical from basolateral surface, whereas subconfluent T84 cells are not polarized and are thus without apical and basolateral segregation. This is similar to the condition of cultured H4 cells. As shown in Fig. 2, subconfluent, nonpolarized T84 cells demonstrated a significantly higher CT uptake than confluent, polarized T84 cells. However, there was no significant difference in the CT-induced cAMP response between these two cell types. This result is consistent with a previous report that, when CT is applied basolaterally, T84 cells demonstrated a higher CT uptake but not a greater secretory response compared with apical application of CT (19). In contrast, HC-treated H4 cells demonstrated a lower rate of CT uptake and a significantly lower cAMP response than H4 cells alone.

Other factors that modulate CT activation of Gs_α. First, using an in vitro ADP-ribosylation assay, we examined CT-catalyzed Gs_α ribosylation in H4 and T84 cells compared with that reported for pre- and postweaned rat small intestinal enterocytes. Consistent with the previous report (41), we found significantly more Gs_α ribosylation in pre- compared with postweaned and adult rat enterocytes (data not shown). However, using cell lysates in a cell-free ribosylation assay, we were unable to detect Gs_α ribosylation in H4 cells, whereas we were able to observe a ribosylation of Gs_α in T84 cells. These observations suggested that the CT-A subunit is capable of ADP-ribosylation of substrate analogs in vitro. However, it has been known that a number of additional membrane proteins and intracellular factors are needed to promote or augment the enzymatic activity in vivo (35). Accordingly, we next investigated whether the addition of rat brain extract that is enriched with ADP-ribosylation factors could facilitate ADP-ribosylation of Gs_α as described in Ref. 29. Increased levels of Gs_α-ribosylation in H4 and T84 (to a lesser degree) cells were noted when rat brain extract was added to the assay mixture (Fig 3*A*). This result suggested that cytosolic/membrane factors in rat brain extract facilitated the CT-mediated ADP-ribosylation of Gs_α in immature human enterocytes. Since newly identified ARFs have been known to facilitate ADP-ribosylation of Gs_α by CT in live cells (35), we next began to examine the role of ARFs in the CT response in H4 vs. T84 cells.

To examine whether ARFs were involved in the CT response, we used an ARFI to block its function. We observed that the ARF inhibitor significantly diminished the CT-induced cAMP response in H4 cells but not in T84 cells, suggesting a possible role of ARFs in mediating the CT response in immature enterocytes (Fig. 3*B*). In mammalian cells, there are six known ARF isoforms, which belong to three classes: ARF1, 2, and 3; ARF 4 and 5; and ARF6. To identify which class/classes ARFs were involved in CT activation of Gs_α, we transfected H4 and T84 cells with siRNAs targeting human ARF genes 1, 3, 4, and 6 before exposure to CT. As shown in Fig. 3*C*, a knockdown of ARF1 and ARF6 significantly inhibited the CT-induced cAMP response in H4 but not in T84 cells. In contrast, neither ARF3 nor ARF4 knockdowns exhibited an effect on the CT response in either cell (Fig. 3*C*). Our RT-PCR data indicated that there is little ARF3 and ARF4 expression compared with ARF1 and 6 expression in H4 and T84 cells (Fig. 3*D*), and this might be the reason that we were not able to observe any difference in ARF3/4 knockdown experiments.

We next examined whether ARFs play a role in either CT uptake or Gs_α activation in H4 cells. Western blot analysis demonstrated that knockdown of ARF1 or ARF6 using siRNA blocked CT-catalyzed ADP-ribosylation of Gs_α compared with cells either maintained in media alone or transfected with control (scrambled) siRNA (Fig. 4). In these experiments, we demonstrated that knockdown of ARF1 and 6 proteins by siRNA (shown in Fig. 4*A*) downregulated CT-mediated Gs_α activation. This is indicated by an absence of retarded migration of the 45-kDa Gs_α isoform on the Western blot as shown in Fig. 4*B*. In addition, knockdown of ARF6 also decreased CT uptake in H4 cells. In contrast, transfection of ARF1 siRNA had no effect on CT uptake (Fig. 4*C*).

ARFs have been shown to be involved in the formation of clathrin-coated vesicles (44) and vesicular transport from the ER to the Golgi (49). Accordingly, we next examined the interaction of ARF1/ARF6 with the CT-endocytosis machinery. H4 cells were transfected with a HA-tagged full-length ARF1/ARF6 for 72 h before exposure to CT. Total cell lysates were immunoprecipitated with an anti-HA antibody before being fractionated on a SDS-PAGE and probed with an anti-Cla-HC antibody. Western blot analysis showed a coprecipitation of clathrin and ARFs in H4 cells that have been exposed

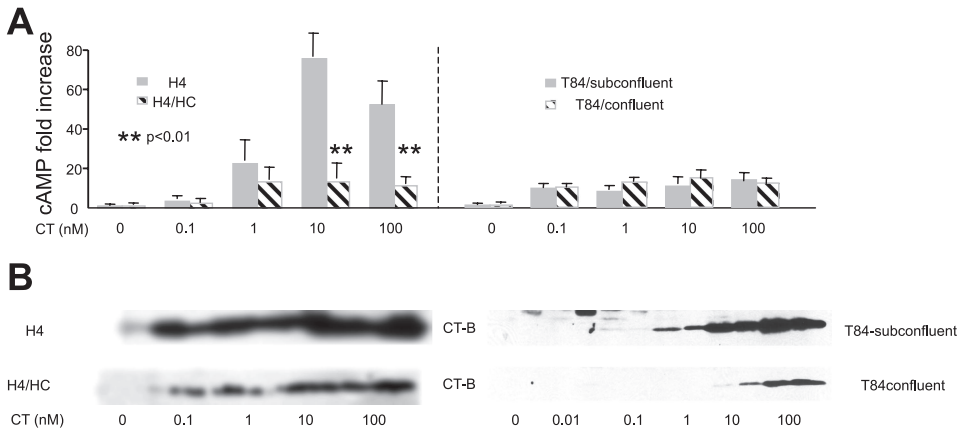

Fig. 2. CT dose responsiveness differs in immature vs. mature enterocytes. *A*: cellular accumulation of cAMP in response to different doses of CT (0.1–100 nM) was monitored in H4 cells with or without pretreatment with HC and T84 cells under subconfluent and confluent/polarized conditions. The results are presented as picomoles per milligram total cellular protein (pmol/mg). The data are representative of 3 experiments. *B*: Western blot analysis of CT uptake in H4 vs. T84 cells under undifferentiated and differentiated conditions. Western blot depicting an expected 11-kDa band (CTB) on a 10–20% SDS-PAGE.

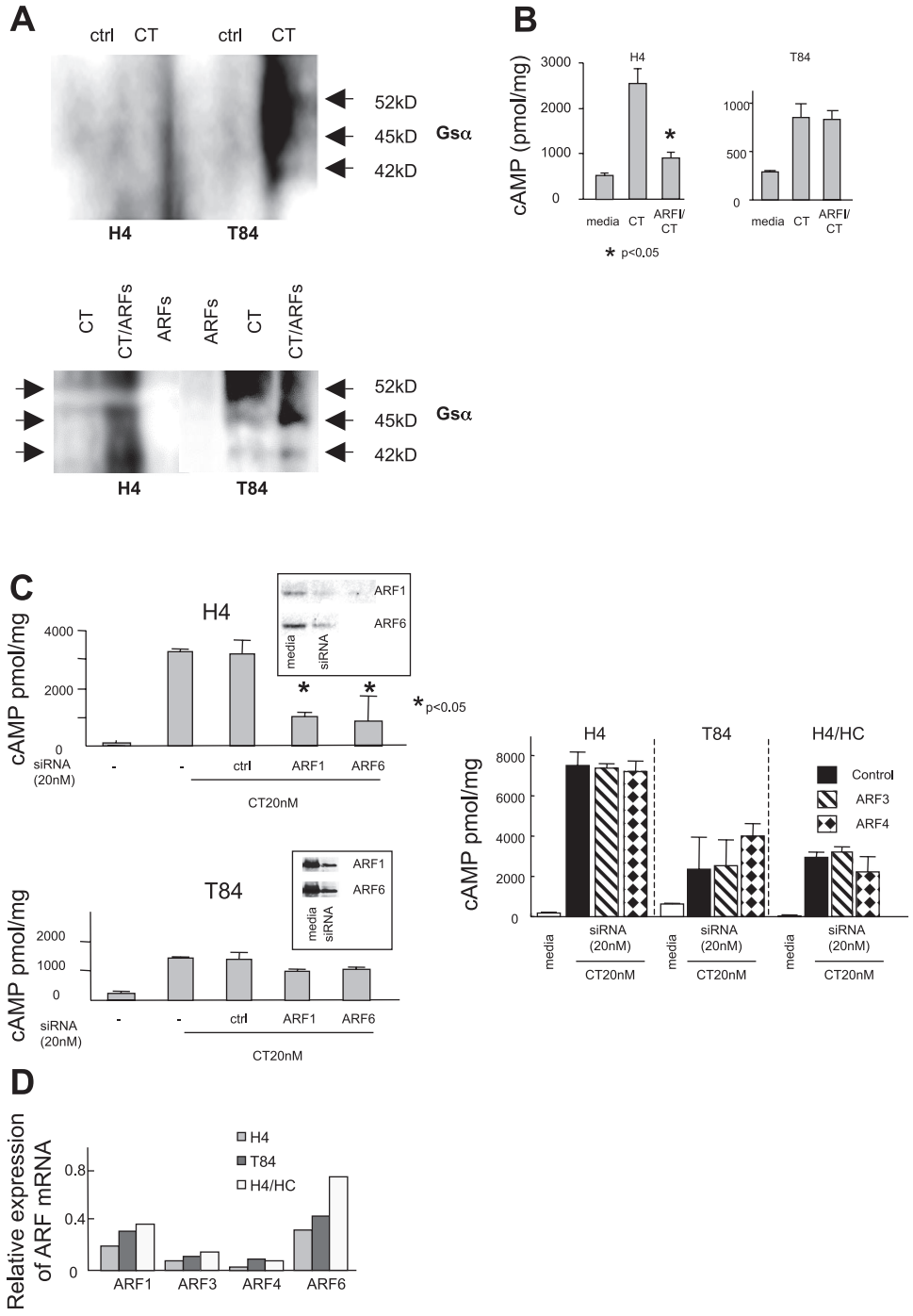

Fig. 3. The effect of ADP-ribosylation factors (ARFs) on the CT-induced cAMP response in H4 vs. T84 cells. *A*: in vitro ADP ribosylation of Gsa by CT in H4 vs. T84 cells. Cell lysates (25 μg) were incubated with γ-32P-NAD with CT or control. Black arrows indicate the ribosylated Gs_α isoforms (1, 2 and 3) at molecular weight of 42, 45, and 52 kDa in T84 cells, but not seen in H4 cells. Addition of ARFs in rat brain extract (Calbiochem) increased CT-catalyzed Gs_α ribosylation in H4 and T84 cells. *B*: H4 and T84 cells were pretreated with ARF inhibitory peptide (ARFI) before CT exposure. Cellular cAMP levels were measured and presented as means ± SE (n = 6). *C*: ARF knockdown affects the CT-induced cAMP response in H4 but not in T84 cells. H4 and T84 were transfected with siRNA targeting ARF1, 3, 4, and 6 or control (scrambled RNA) before CT exposure. Cellular accumulation of cAMP was measured and expressed as means ± SE (n = 6). *Inset*: knockdown efficiency of ARF1 and ARF6 in H4 and T84 cells, respectively. ARF3 or ARF4 knockdown had no effect on the CT response in H4 and T84 cells (real-time PCR cannot detect ARF3/4 mRNA expression after siRNA treatment). *D*: real-time PCR of ARF1, 3, 4, and 6 mRNAs expression in H4 vs. T84 cells normalized by GAPDH expression.

to CT at 37°C (Fig. 5*A*). Since the EGF receptor (EGFR) is known to be internalized via a clathrin-mediated endocytosis upon ligand binding, we used EGFR endocytosis to examine the specificity of ARFs in regulating CT endocytosis and trafficking. As shown in Fig. 5*B*, there was no coprecipitation of clathrin and ARFs in H4 cells that have been incubated with EGF, suggesting that the ARF effect is specific for the CT response.

Collectively, these data suggest that ARFs might affect the CT response through complementary mechanisms, e.g., ARF1 had its effect principally on CT trafficking and signaling (Figs. 4*B* and 5*A*), whereas ARF6 plays a major role in CT endocytosis (Figs. 4*C* and 5*A*).

In summary, the CT response via Gs_α signaling in fetal enterocytes may differ from that of mature enterocytes, and the difference may be related to ARF interaction with the CT-signaling process.

HC affects CT activation of Gs_α in H4 cells. In a previous study, we have shown that a known breast milk trophic factor, HC, can alter the excessive secretory response to CT in immature human fetal enterocytes (23) and can induce a maturation change in endocytosis (25). We concluded that this

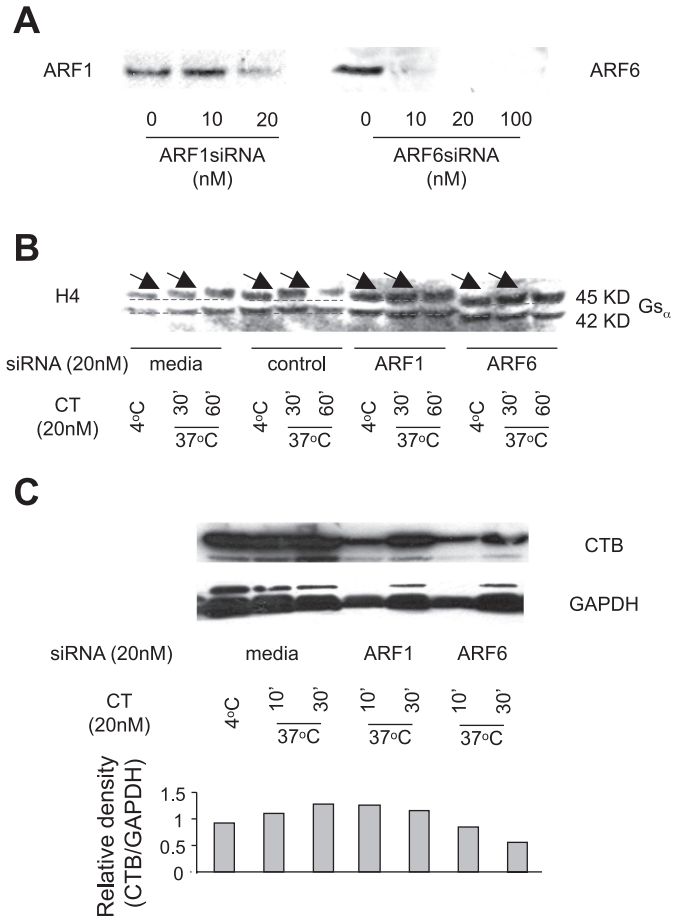

Fig. 4. Downregulation of ARF1 or ARF6 affects the CT response in H4 cells. *A*: efficiency of ARF1/6 knockdown by siRNAs on Western blot. *B*: effect of ARF1/6 knockdown on CT-induced Gs_α ribosylation in H4 cells. The black arrows indicate the CT-catalyzed ribosylation of Gs_α isoform (45 kDa, upshifted) in H4 cells treated with either media alone or control siRNA. Dashed arrows indicate lack of Gs_α isoform upshift in H4 cells transfected with either ARF1 or ARF6 siRNA. *C*: effect of ARF1/6 gene knockdown on CT uptake in H4 cells. Western blots depicting the expected 11-kDa band probed with anti-CTB antibody (1:5,000).

change in CT endocytosis was partially attributable to a HC-induced CT-GM1 lipid raft association through maturational changes in the plasma membrane phospholipid composition of immature enterocytes (24). In this study, we investigated whether HC affected the developmental control of CT-induced Gs_α activation. H4 cells were preincubated with HC (1 μM) for 24 h or 5 days before CT exposure. As shown in Fig. 6*A*, pretreatment of H4 cells in HC (H4/HC) induced a Gs_α lipid raft association when fractionated on a 5–30% sucrose gradient column. After H4/HC cells were challenged with CT, Western blot analysis demonstrated a decrease in Gs_α activation only in cells that have been pretreated with HC for 5 days compared with H4 cells grown either in media or in HC for 24 h (Fig. 6*B*). This lack of activation was not due to a decrease in Gs_α mRNA or protein expression. The gene expression analysis and real-time RT-PCR in H4 cells preincubated with HC for various lengths of time (12, 24, and 48 h to 7 days) demonstrated a slight increase of mRNA expression after HC treatment (Fig. 1*A*).

Next, we examined whether pretreatment of H4 cells with HC (5–7 days) could affect the role of ARFs in the CT response. We transfected H4/HC cells with siRNAs targeting either ARF1 or ARF6. In contrast to data shown in Fig. 3*B*, HC pretreatment diminished the ARF-dependent CT response measured as CT-induced cAMP production in these cells (Fig. 6*C*). Western blot analysis demonstrated a sufficient knockdown of ARF1/6 proteins in H4/HC cells (Fig. 6D).

DISCUSSION

CT is responsible for the devastating diarrheal syndrome characteristic of cholera. CT-induced secretory diarrhea occurs more commonly and severely in young infants than in older children and adults. It is the best-characterized disease mediated by the Gs_α/cAMP signaling pathway. Previous studies from our laboratory have shown an increased cAMP response to CT in the small intestine of suckling compared with mature rats (43). This increased sensitivity of AC to CT is largely due to higher Gs_α mRNA expression and increased ADP-ribosylation of one or both Gs_α isoforms in the preweaned rat small intestine (41). We previously reported that immature human enterocytes exhibit an excessive secretory response to CT, and this response is, in part, due to a developmental difference in CT endocytosis (25). However, little is known about the developmental regulation of the CT signaling leading to Gs_α activation in immature human enterocytes. In this study, we have examined CT signaling via Gs_α activation and provide evidence to suggest that the molecular basis for this developmental change in host responsiveness might be related to a difference in the CT-endocytosis machinery utilized by immature human enterocytes. In addition, we provide evidence for a

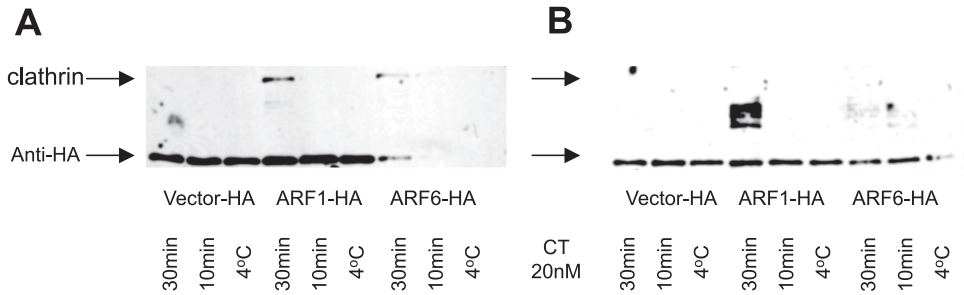

Fig. 5. ARF1 and ARF6 interact with clathrin in the CT-mediated signaling process. H4 cells were transfected with hemaglutinin (HA)-tagged ARF1 and ARF6 expression vectors before being immune precipitated with an anti-HA antibody, resolved on SDS-PAGE, and probed with an anti-clathrin heavy-chain (Cla-HC) antibody (1:1,000). *A*: coprecipitation of ARF1/6 with Cla-HC after CT exposure in H4 cells. A Western blot depicts an expected Cla-HC band at 180 kDa on a 4–20% linear gradient gel. *B*: H4 cells were incubated with 100 ng of EGF. There is no EGF receptor-induced coimmunoprecipitation of ARF1/6 with clathrin.

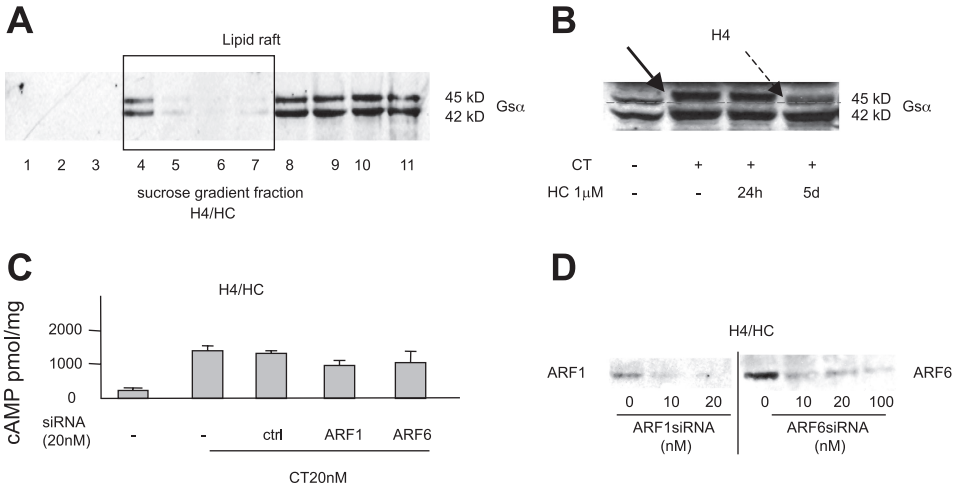

Fig. 6. The intestinal trophic factor, HC, affects the CT-mediated Gs_α response and ARF function in H4 cells. *A*: H4 cells were preincubated with HC for 5 days, and Gs_α distribution was examined on a sucrose gradient column (5–30%). *Fractions 4–7* contain lipid rafts. *B*: H4 cells were pretreated with HC for 1 or 5 days before CT exposure. A Western blot depicts the expected Gs_α isoforms probed with an anti-Gs_α antibody. The black arrows indicate Gs_α ribosylation (upshift of 45 kDa Gs_α isoform), and the dashed arrows indicate a diminished Gs_α activation after preincubation with HC for 5 days. Twenty-four-hour pretreatment has no effect on the CT response in H4 cells. *C*: H4/HC (5 days) cells were transfected with siRNA targeting ARF1, ARF6, or control before CT exposure. Intracellular cAMP elevation was measured and expressed as means ± SE. *D*: ARF1/6 gene knockdown efficiency in H4/HC cells monitored by Western blotting.

major role that ARF1/6 play in CT trafficking and signaling in immature human enterocytes.

Gs_α is ubiquitously expressed in various tissues, and its cellular distribution changes under a wide range of metabolic conditions such as cellular differentiation, ontogenic development, aging, and various adaptive processes (10, 11, 45). Here, our results have shown a higher level of Gs_α protein expression as well as in vitro CT-catalyzed ADP-ribosylation of Gs_α in mature compared with immature enterocytes. However, this observation was in contrast with the observed increase of Gs_α ADP-ribosylation in intact H4 vs. T84 cells. This inconsistency between cell-free and intact cell observations suggests that additional factor(s) maybe involved in CT signaling leading to Gs_α activation. Furthermore, we compared the difference in the CT response in subconfluent vs. confluent (nonpolarized vs. polarized) T84 cells. Interestingly, subconfluent T84 cells showed an increase in CT uptake, yet no difference in the CT-induced cAMP response compared with confluent cells. This observation is in contrast to the observations made in H4 cells in which pretreatment with HC significantly decreased CT uptake (25) and diminished the cAMP response, respectively. Taken together, these data suggest that the observed difference in the CT response between H4 and T84 cells is likely attributable to a posttranscriptional modification of Gs_α that leads to its excessive activation.

Recent developments have provided interesting speculations about the possible cellular or molecular basis for regulating CT induced Gs_α signaling in developing human enterocytes. First, compartmentalized (segregated) Gs_α may prevent CT access. A striking enrichment of Gs_α in the Triton X-100-insoluble and lipid-raft fraction of the cell membrane was found in T84 and H4/HC cells, which was absent in H4 cells. Secondly, the involvement of $G_{\beta\gamma}$ subunits in Gs_α activation may differ. It has been proposed that the $G_{\beta\gamma}$ subunits are a membrane anchor for Gs_α, and dissociation/release of $G_{\beta\gamma}$ from Gs_α leads to activation of AC and elevation of cAMP (15, 48). In a cyc-s49 system, both Gs_α and $G_{\beta\gamma}$ had to be present in the membrane, and they had to be able to form a heterotrimer in order for CT to ADP-ribosylate Gs_α (15). Moreover, it was reported that $G_{\beta\gamma}$ plays an important role in targeting Gs_α to the plasma membrane and is essential for binding and activating of the heterotrimeric G proteins by receptors in HEK293 cells (12, 13). In H4 cells, $G_{\beta\gamma}$ subunits were mostly present in the Triton X-100-soluble membrane fraction similar to the distribution of Gs_α (Fig. 1). We provide evidence that, upon CT exposure, there was a decrease in membrane $G_{\beta\gamma}$ and an increase in CT-induced Gs_α activation in H4 cells. However, in T84 cells $G_{\beta\gamma}$ subunits were largely present in the Triton X-100-soluble membrane fraction, a location segregated from the lipid raft-associated Gs_α. In this manner, pretreatment of H4 cells with HC can induce a maturational change in the plasma membrane (24) and an increased lipid raft association with Gs_α that results in a decreased CT response. These observations suggest that in human fetal enterocytes the lack of lipid-raft association might provide an easier access for CTA1 to interact with Gs_α. Furthermore, the association with $G_{\beta\gamma}$ subunits in the plasma membrane may facilitate the CT-catalyzed ADP-ribosylation of Gs_α, leading to AC activation and cAMP elevation. In contrast, in T84 cells, Gs_α proteins are enriched in lipid rafts and segregated from $G_{\beta\gamma}$. This structure might restrict the access of CTA1 to Gs_α, resulting in a reduced CT response. However, further studies are needed to determine whether interaction between Gs_α and $G_{\beta\gamma}$ at the plasma membrane and subsequent dissociation of $G_{\beta\gamma}$ is important for Gs_α activation and the CT response in human enterocytes.

CT-catalyzed ADP-ribosylation of Gs_α and activation of AC are enhanced by a family of soluble or membrane factors termed ARFs (35, 46). Previous studies have supported the hypothesis that ARFs are allosteric activators of CTA1 (35). However, recent studies have demonstrated that ARF proteins are not only activators of CT but also critical components of intracellular vesicular transport processes (30, 36). Here, we provided evidence that ARF1 and as ARF6 are required for CT uptake and signaling in human fetal enterocytes (Fig. 4). Both ARF1 and ARF6 have been shown to participate in clathrin-mediated vesicle trafficking events, but they function at distinctly different steps in membrane trafficking (3, 44). For instance, ARF1 can function through the assembly of clathrin-coated proteins in the ER/Golgi membrane, in ER-Golgi and intra-Golgi transport, or in transport vesicles from the trans-Golgi network (28, 42). In contrast, ARF6 can play an important role in clathrin-mediated endocytosis (1, 33). Their function in membrane trafficking is the recruitment of membrane coat proteins including coatomer protein (22), clathrin, and

clathrin adaptor proteins such as AP1, AP2, AP3, and AP4 complexes (17, 26, 32, 34). Some studies have shown that ARFs can also activate phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase), an enzyme that generates PtdIns(4,5)P2 (3). Phosphoinositols [PtdIns(4,5)P2 and PtdIns(3,4,5)P3] have been shown to affect clathrin-mediated membrane trafficking (7, 8, 47). However, further studies are needed to clarify the possible interactions between ARF6 and clathrin-coated vesicles to elucidate the mechanism by which ARF6 regulates clathrin-mediated vesicular trafficking either by being directly involved in coat formation, by recruitment of clathrin adaptor protein (such as AP2), or by interaction with phospholipids particularly, PtdIns(4,5)P2 and PtdIns(3,4,5)P3.

ARF1 is required in both clathrin-dependent and -independent vesicular transport in the Golgi (26). We have shown in our preliminary results that ARF1 is required for CT-mediated AC activation in H4 and to a lesser degree in T84 cells (Fig. 2) because knocking down ARF1 expression in H4 cells resulted in a significantly diminished CT-induced cAMP response without affecting CT uptake. In contrast, there is only a slight decrease of cAMP response in T84 cells. It has been reported that ARF1 is required for the formation of clathrin-coated vesicles at the Golgi level in cells, whereas ARF6 has been involved in plasma membrane trafficking. This is consistent with our findings that, in human fetal immature enterocyte, ARF1 and ARF6 function at different levels in regulating clathrin-dependent CT trafficking or endocytosis, respectively (Figs. 4 and 5).

In this study, we show that an immature Gs_α response exists in immature human fetal enterocytes leading to an excessive AC activation and cAMP response. We provide evidence that membrane/cellular factors, particularly ARF1 and ARF6, contribute to a critical role in regulating clathrin-mediated CT uptake, intracellular trafficking, and/or CT signaling in these immature enterocytes. Moreover, we provide preliminary evidence that trophic factors may modulate the immature Gs_α response toward a more mature response. It is noteworthy that in human enterocytes there is a developmental alteration in the plasma membrane phospholipid content (24). A maturation of plasma membrane lipid composition may result in compartmentalization and distribution changes in many cellular factors such as heterotrimeric G proteins Gs_α and $\beta\gamma$ subunits, as well as ARF family proteins, leading to changes in CT endocytosis, intracellular trafficking, and eventually in signal transduction and effector responses. Collectively, these findings support our hypothesis that there is a developmentally controlled intestinal cellular response to bacterial exotoxins involving complex cellular events. One caveat in interpreting our studies is that T84 cells, which are an adult human colonic carcinoma epithelial cell line, do not perfectly model normal mature enterocytes. Therefore, it would be inappropriate to use T84 cells as a comparison for all factors that influence intestinal immaturity. However, previous studies comparing intestinal development in H4 cells and T84 cells have been validated in multiple systems including suckling rats, xenografted human fetal small intestine, and primary cultures of freshly isolated human small intestinal epithelial cells. We believe these studies will further our understanding of the development of host defense during postpartum gut maturation. A better understanding of these mechanisms may help to plan strategies for prevention.

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REFERENCES

1. **Altschuler Y, Liu SH, Katz L, Tang K, Hardy S, Brodsky F, Apodaca G, Mostov K.** ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J Cell Biol* 147: 7–12, 1999.
2. **Badizadegan K, Dickinson BL, Wheeler HE, Blumberg RS, Holmes RK, Lencer WI.** Heterogeneity of detergent-insoluble membranes from human intestine containing caveolin-1 and ganglioside GM1. *Am J Physiol Gastrointest Liver Physiol* 278: G895–G904, 2000.
3. **Brown FD, Rozelle AL, Yin HL, Balla T, Donaldson JG.** Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J Cell Biol* 154: 1007–1018, 2001.
4. **Chu SW, Walker AW.** Bacterial toxin interaction with the developing intestine: a possible explanation for toxogenic diarrhea of infancy. *Gastroenterology* 104: 916–925, 1993.
5. **Chu W, Ely IG, Walker WA.** Age and cortisone alter host responsiveness to cholera toxin in the developing gut. *Am J Physiol Gastrointest Liver Physiol* 256: G220–G226, 1989.
6. **Claud EC, Lu L, Anton PM, Savidge T, Walker AW, Cherayil BJ.** Developmentally regulated IκB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc Natl Acad Sci USA* 101: 7404–7408, 2004.
7. **Corvera S, D'Arrigo A, Stenmark H.** Phosphoinositides in membrane traffic. *Curr Opin Cell Biol* 11: 460–465, 1999.
8. **Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P.** Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell* 99: 179–188, 1999.
9. **Dominguez P, Barros F, Lazo PS.** The activation of adenylate cyclase from small intestinal epithelium by cholera toxin. *Eur J Biochem* 146: 533–538, 1985.
10. **Duman RS, Saito N, Tallman JF.** Development of [beta]-adrenergic receptor and G protein messenger RNA in rat brain. *Mol Brain Res* 5: 289–296, 1989.
11. **Espinasse I, Iourgenko V, Defer N, Samson F, Hanoune J, Mercadier JJ.** Type V, but not Type VI, adenylyl cyclase mRNA accumulates in the rat heart during ontogenic development. Correlation with increased global adenylyl cyclase activity. *J Mol Cell Cardiol* 27: 1789–1795, 1995.
12. **Evanko DS, Thiyagarajan MM, Takida S, Wedegaertner PB.** Loss of association between activated G[alpha]q and G[beta][gamma] disrupts receptor-dependent and receptor-independent signaling. *Cell Signal* 17: 1218–1228, 2005.
13. **Evanko DS, Thiyagarajan MM, Wedegaertner PB.** Interaction with Gbeta gamma is required for membrane targeting and palmitoylation of Galpha s and Galpha q. *J Biol Chem* 275: 1327–1336, 2000.
14. **Field M, Rao MC, Chang EB.** Intestinal electrolyte transport and diarrheal disease. *N Engl J Med* 321: 879–883, 1989.
15. **Ganpat MM, Nishimura M, Toyoshige M, Okuya S, Pointer RH, Rebois RV.** Evidence for stimulation of adenylyl cyclase by an activated Gs heterotrimer in cell membranes: an experimental method for controlling the Gs subunit composition of cell membranes. *Cell Signal* 12: 113–122, 2000.
16. **Gill DM, King CA.** The mechanism of action of cholera toxin in pigeon erythrocyte lysates. *J Biol Chem* 250: 6424–6432, 1975.
17. **Krauss M, Kinuta M, Wenk MR, De Camilli P, Takei K, Haucke V.** ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type I[gamma]. *J Cell Biol* 162: 113–124, 2003.
18. **Lencer WI.** Microbes and microbial toxins: paradigms for microbial-mucosal interactions. V. Cholera: invasion of the intestinal epithelial barrier by a stably folded protein toxin. *Am J Physiol Gastrointest Liver Physiol* 280: G781–G786, 2001.
19. **Lencer WI, Charlene D, Neutra MR, Madara JL.** Mechanism of cholera toxin action on a polarized human intestinal epithelial cell line: role of vesicular traffic. *J Cell Biol* 117: 1197–1209, 1992.
20. **Lencer WI, Chu SW, Walker WA.** Differential binding kinetics of cholera toxin to intestinal microvillus membrane during development. *Infect Immun* 55: 3126–3130, 1987.

21. **Lencer WI, Tsai B.** The intracellular voyage of cholera toxin: going retro. *Trends Biochem Sci* 28: 639–645, 2003.
22. **Liu W, Duden R, Phair RD, Lippincott-Schwartz J.** ArfGAP1 dynamics and its role in COPI coat assembly on Golgi membranes of living cells. *J Cell Biol* 168: 1053–1063, 2005.
23. **Lu L, Baldeon ME, Savidge T, Pothoulakis C, Walker WA.** Development of microbial-Human enterocyte interaction: cholera toxin. *Pedatr Res* 54: 212–218, 2003.
24. **Lu L, Bao Y, Khan A, Goldstein AM, Newburg DS, Quaroni A, Brown D, Walker WA.** Hydrocortisone modulates cholera toxin endocytosis by regulating immature enterocyte plasma membrane phospholipids. *Gastroenterology* 135: 185–193; e181, 2008.
25. **Lu L, Khan S, Lencer WI, Walker WA.** Endocytosis of cholera toxin by human enterocytes is developmentally regulated. *Am J Physiol Gastrointest Liver Physiol* 289: G332–G341, 2005.
26. **Mariadason JM, Nicholas C, L'Italien KE, Zhuang M, Smartt HJM, Heerdt BG, Yang W, Corner GA, Wilson AJ, Klampfer L, Arango D, Augenlicht LH.** Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. *Gastroenterology* 128: 1081–1088, 2005.
27. **McKenzie FR.** *Basic Techniques to Study G-protein Function.* Oxford, UK: New York IRL, 1992.
28. **Memon AR.** The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants. *Biochim Biophys Acta* 1664: 9–30, 2004.
29. **Moss J, Vaughan M.** Activation of cholera toxin and Escherichia coli heat-labile enterotoxins by ADP-ribosylation factors, a family of 20 kDa guanine nucleotide-binding proteins. *Mol Microbiol* 5: 2621–2627, 1991.
30. **Moss J, Vaughan M.** Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J Biol Chem* 21: 12327–12330, 1995.
31. **Nanthakumar NN, Fusunyan DR, Sanderson RI, Walker WA.** Inflammation in the developing human intestine: a possible pathophysiologic basis for necrotizing enterocolitis. *Proc Natl Acad Sci USA* 97: 6043–6048, 2000.
32. **Ooi CE, Dell'Angelica EC, Bonifacino JS.** ADP-ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. *J Cell Biol* 142: 391–402, 1998.
33. **Osada H, Watanabe Y, Fujii TK, Tsunoda I, Tsubata K, Satoh K.** Stimulation of early embryonic development in cattle by coculture with surfactant. *J Assist Reprod Genet* 16: 310–314, 1999.
34. **Paleotti O, Macia E, Luton F, Klein S, Partisani M, Chardin P, Kirchhausen T, Franco M.** The small G-protein Arf6GTP recruits the AP-2 adaptor complex to membranes. *J Biol Chem* 280: 21661–21666, 2005.
35. **Price RS, Nightingale M, Tsai SC, Williamson KC, Adamik R, Chen HC, Moss J, Vaughan M.** Guanine nucleotide-binding proteins that enhance choleragen ADP-ribosyltransferase activity: nucleotide and deduced amino acid sequence of an ADP-ribosylation factor cDNA. *Proc Natl Acad Sci USA* 85: 5488–5491, 1988.
36. **Randazzo PA, Terui T, Sturch S, Kahn RA.** The amino terminus of ADP-ribosylation factor (ARF) 1 essential for interacting with Gs and ARF GTPase-activating protein. *J Biol Chem* 269: 29490–29494, 1994.
37. **Raufman JP.** Cholera. *Am J Med* 104: 386–394, 1997.
38. **Sack DA, Sack BR, Nair BG, Siddique AK.** Cholera. *Lancet* 363: 223–233, 2004.
39. **Sanderson IR, Ezzell RM, Kedinger M, Erlanger M, Xu Z, Pringault E, Leon-Robine S, Louvard D, Walker WA.** Human fetal enterocytes in vitro: modulation of the phenotype by extracellular matrix. *Proc Natl Acad Sci USA* 93: 7717–7722, 1996.
40. **Sanderson IR, Walker WA.** Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). *Gastroenterology* 104: 622–639, 1993.
41. **Sanderson IR, Xu Z, Chu SW, Xie QY, Levine L, Walker WA.** Developmental differences in the stimulatory GTP binding protein subunit (Gsα) for adenylate cyclase in the rat small intestine. *Gut* 38: 853–858, 1996.
42. **Schwaninger R, Plutner H, Bokoch GM, Balch WE.** Multiple GTP-binding proteins regulate vesicular transport from the ER to Golgi membranes. *J Cell Biol* 119: 1077–1096, 1992.
43. **Seo JK, Chu SW, Walker WA.** Development of intestinal host defense: an increased sensitivity in the adenylate cyclase response to cholera toxin in suckling rats. *Pediatr Res* 25: 225–227, 1989.
44. **Stamnes MA, Rothman JE.** The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell* 73: 999–1005, 1993.
45. **Trevor Young L, Warsh JJ, Li PP, Siu KP, Becker L, Gilbert J, Hornykiewicz O, Kish SJ.** Maturational and aging effects on guanine nucleotide binding protein immunoreactivity in human brain. *Dev Brain Res* 61: 243–248, 1991.
46. **Tsai SC, Adamik R, Tsuchiya M, Chang PP, Moss J, Vaughan M.** Differential expression during development of ADP-ribosylation factors, 20-kDa guanine nucleotide-binding protein activators of cholera toxin. *J Biol Chem* 266: 8213–8219, 1991.
47. **Vallis Y, Wigge P, Marks B, Evans PR, McMahon HT.** Importance of the pleckstrin homology domain of dynamin in clathrin-mediated endocytosis. *Curr Biol* 9: 257–263, 1999.
48. **Warner DR, Okuya S, Rebois RV.** Altered Gs[alpha] N-terminus affects Gs activity and interaction with the G[beta][gamma] subunit complex in cell membranes but not in solution. *Cell Signal* 8: 43–53, 1996.
49. **Waters MG, Griff IC, Rothman JE.** Proteins involved in vesicular transport and membrane fusion. *Curr Opin Cell Biol* 3: 615–620, 1991.
50. **Wolf AA, Jobling MG, Wimer-Mackin S, Ferguson-Maltzman M, Madara JL, Holmes RK, Lencer WI.** Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J Cell Biol* 141: 917–927, 1998.